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In vitro Evaluation of *Aristolochia indica* for its Anti-inflammatory, Antidiabetic and Anticancer Efficacy

Janani, N and Revathi, K

[1]Research Scholar, [2]Principal and Head, Department of Zoology, Ethiraj College for Women, Chennai-8, Tamilnadu, India.

*Corresponding author: *reva63@rediffmail.com*

Abstract

Aristolochia indica is an important medicinal herb and it is an original of Indian subcontinent and has become naturalized in the tropical and sub-tropical areas around the world. The plant is usually gathered from the wild and is used locally in traditional medicine. It is sometimes cultivated for medicinal use in India. In the indigenous system of medicine, the plant was used as purgative, antipyretic & anti-inflammatory agents. Hence the present study was carried out to assess the therapeutic potential of the dried powder of whole plant of *Aristolochia indica.* Further, the work may be suggested to perform the isolation and characterization and mechanism of action of the active compound which may be responsible for the biological activities.

Keywords: *Aristolochia indica*, HRBC Membrane stabilization. Alpha Amylase, Alpha Glucosidase, Anticancer, HepG2 Cancer cell lines.

1. Introduction

Tissues of many plants species contain secondary metabolites with the potential to combat disease-causing micro-organisms. These compounds include glycosides, saponins, flavonoids, steroids, tannins, alkaloids and terpenes (EL-Kamali et al, 2010). Extracts of different plant organs, including roots, leaves, bark, flowers, fruits and seeds, may contain distinct phytochemicals with activity against bacterial or fungal pathogens (Tiwari et al, 2011). In folk medicine, a single plant species is often used to treat more than one type of disease or infection (Chandran et al, 2013). Extracts of plants with a history of traditional use should be tested using modern methods for activities against human pathogens, with the aim of discovering potential new drugs.

Inflammatory diseases comprising various kinds of rheumatism are common all over the world (Chamundeeswari et al, 2003). Although rheumatism is the oldest known human disease, limited progress has been made for its permanent treatment. Non-steroidal anti-inflammatory drugs (NSAIDs) are being used to cure and control inflammation, fever and pain. However, their use has not been therapeutically efficacious in all types of inflammations. Furthermore, the use of NSAIDs can cause adverse side-effects leading to hemorrhage and ulcers (Qin et al,2011).

Cancer is a deadly disease and about one in four people will get it in some form during their lifetime; at the present time, about one in five of all deaths are due to cancer (Chhajed et al,2012)Normal diploid human cells multiply for a finite number of generations and then enter a state of replicative senescence, but cancer cells can proliferate indefinitely (Rao et al, 2007) About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these,56%of the cases and 64% of the deaths occurred in the economically developing world.

Based on the fact that some species of the genus Aristolochia have been reported to possess anticancer activity this study was designed to investigate the anti inflammatory, antidiabetic and anticancer activity of *A.indica* using *in vitro* methods.

2. Materials and Methods

2.1 Collection of plant material

The plant *Aristolochia indica* was collected from the local gardens of Chennai during the period from December 2016 to January 2017. Then it was preserved in the laboratory for future reference.

2.2 Preparation of *Aristolochia indica* powder

The plant was thoroughly washed using distilled water to remove soil debris. The whole plant used for extraction was shade dried and powdered using mechanical grinder. The whole plant dried powder of *Aristolochia indica* was obtained by sieving and collected in two air tight container. Dried powder of the whole plant of *Aristolochia indica* in one container was used for determining the physicochemical analysis and other was used for the aqueous and methanol extraction.

2.3 In vitro anti-inflammatory assays

The test samples at different concentrations in the range of 100 to 500 µg/ ml were taken in the test tubes. 1% of aqueous solution of bovine albumin fraction was added to the test tube containing the test samples. The reaction mixture was mixed well and the pH was adjusted by adding the small amount of 1N HCL. Test tubes were incubated at 37° C for 20 minutes and then heated at 57° C for 20 minutes. Test tubes were cooled and the absorbance was measured at 660nm. The samples were prepared in triplicates for each analysis and the absorbance was obtained. Diclofenac sodium was used as a standard drug of varied concentration (100-500µg/ ml) to determine the absorbance. Inhibition percentage of protein denaturation was calculated as follows: % inhibition = (Abs control – Abs sample) X 100 / Abs control.

Preparation of Human Red Blood cells (HRBCs) suspension

Fresh human blood (10 ml) was collected, which is then transferred to the heparinized centrifuged tubes. The blood containing tubes were centrifuged at 3000 rpm for 10 minutes. The packed cells were washed three times with normal saline solution and reconstituted as a 10% v/v suspension with normal saline (Shinde *et al.*, 1999).

The methanolic extract was taken separately in the centrifuge tube. 2 ml of reaction mixture contains 1 ml of the test sample and 1ml of 10% RBC suspension Aspirin was used as a reference drug and saline was used in the control tube. All the tubes containing the reaction mixture were incubated at 56°C for 30 minutes and cooled under running tap water. Then the tubes were centrifuged at 2500rpm for 5 minutes. The absorbance of the supernatant solution was estimated by using UV analysis at 560nm.Diclofenac sodium was used as a standard drug of varied concentration (100-500µg/ ml) to determine the absorbance. The experiments were conducted in triplicates (Shinde *et al.*, 1999).Percent of HRBC membrane stabilization or protection was calculated as follows:% of protection = (100 – O.D of drug treated sample – O.D of control) X 100.

2.4 In vitro anti- diabetic assays

Alpha amylase enzyme assay (DNSA method)

Various concentrations of the test sample in the range of 100 – 500 µg/ ml was taken in the test tubes. From this, 500 µl of sample was taken and added to 500 µl of starch solution and kept for 10 minutes. Further, the reaction was initiated by adding the alpha- amylase enzyme solution and the test tubes were incubated at 37° C for 10 minutes. Finally, the reaction was stopped by

adding 1 ml of 3, 5 dinitro salicylic acids, the contents are mixed well and it is kept incubated in the boiling water bath for 5 minutes, and then cooled to room temperature. The reaction mixture was diluted by adding 10 ml of distilled water. The samples were prepared in triplicates for each analysis and the absorbance was obtained. The absorbance was measured at 540 nm. Acarbose was used as standard. The similar procedure was followed for the standard drug Acarbose. The percentage of inhibition was calculated from the following formula: % inhibition = [(O.D of control – O.D of test sample)/ O.D of control] × 100.

Alpha glucosidase enzyme assay

The methanolic extract of *Aristolochia indica* was tested for its alpha glucosidase enzyme activity by the method given by Murugesan *et al.,* (2015).

1 ml of the test sample in the range of 100 – 500 µg/ ml were taken in the test tubes containing 1ml solution of starch substrate (2 % w/v maltose or sucrose) and 16mM of Tris buffer (pH 8.0) then the test tubes were incubated at 37° C for 5 minutes. The reaction was initiated by adding the alpha- glucosidase enzyme solution (IU/ml) and the test tubes were incubated at 35° C for 40 minutes. Finally, the reaction was stopped by adding 2 ml of 6N HCL. The absorbance was measured at 540 nm Acarbose was used as standard. The samples were prepared in triplicates for each analysis and the absorbance was obtained. The similar procedure was followed for the standard drug Acarbose. The percentage of inhibition was calculated from the following formula: % inhibition = [(O.D of control – O.D of test sample)/ O.D of control] × 100.

2.5 In vitro cyto toxicity (MTT) assay

The methanolic extract of *Aristolochia indica* was subjected to *in vitro* cyto toxicity by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay method using human liver cell lines given by Tim Mosmann (1983).
Cell lines

The anticancer activity of methonal extract of *A.india* was performed on HepG2 cell lines obtained from NCCLS Pune, India. MTT assay was performed to determine the cytotoxic property of *A.indica* extract against VERO cell line and cytoprotective role in HepG2 cell lines through MTT assay (*Mosmann et al*.,1983). Stock solutions of *A.indica* extract were prepared in sterile distilled water and diluted to the required concentrations (50, 25, 12.5, 6.25, 3.12, 1.56 mg/ml) using the cell culture medium. Cells (1×10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO_2 incubator for 72 hours. Then, add various concentrations of the samples in 0.1% DMSO incubated for 24hrs at 5 % CO_2 incubator. After removal of the sample solution, 20µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)- 2, 5- diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC^{50}) was determined graphically. The results were expressed as the % cell viability, using the following formula: %Cell viability = A540 of treated cells / A540 of control cells × 100%.

Controls were maintained throughout the experiment. The assay was performed in triplicates for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract. Cells and % viability was plotted against concentration of the plant extract. The maximum concentration of the plant extract that was non toxic to Vero cells but toxic to HepG2 cell lines was recorded as the effective drug concentration

3. Results

3.1 In vitro anti-inflammatory

The present work on *Aristolochia indica* has revealed that no work has been done to show their *in vitro* anti-inflammatory activity. Hence, we have carried out *in vitro* anti-inflammatory activity assays to analyse the ability of the plant extract. The result of the *in vitro* anti-inflammatory activity of methanolic extract of *Aristolochia indica* is summarized in Table 1.

Table 1. *In vitro* anti-inflammatory assays of methanolic extract of *Aristolochia indica*

Concentration (µg/ ml)	STD 250(µg/ ml)	Inhibition of albumin denaturation (%)	Heat induced hemolysis test
100	89.74±0.18	59.18±0.47	63.26±0.39
200		64.07±0.25	67.21±0.14
300		70.66±0.12	75.14±0.03
400		76.08±0.69	77.35±0.34
500		72.17±0.46	77.06±0.04

The albumin denaturation method was used as a convenient tool to analyse the extent of inhibition rendered by the plant extract. It was concluded from the result that the methanolic extract of *Aristolochia indica* inhibited albumin denaturation in a concentration dependent manner. At 100µg/ml, about 59.18% inhibition was observed and further increased to 72.17 % on increasing the concentration to 500µg/ml. The results were compared to the standard drug diclofenac sodium which exhibits 89.74% inhibition. The inhibition of heat induced hemolysis by the methanolic extract of *Aristolochia indica* was tested and the results are given below. The result showed that the methanolic extract of *Aristolochia indica* inhibited heat induced hemolysis in a dose-dependent manner. The extract inhibited 63.26% of hemolysis at 100µg/ml concentration and 77.06% at 500µg/ml which was compared to standard drug as diclofenac sodium 89.74% at 250µg/ml. Therefore, the inhibiting activity of the methanolic extract of the plant was increased with the increasing concentration.

3.2. In vitro anti- diabetic

Table 2 - *In vitro* anti-diabetic assays of methanolic extract of *Aristolochia indica*

Concentration of the test sample (µg/ ml)	α-amylase inhibition assay		α-glucosidase enzyme assay	
	STD (250µg/ ml)	Test sample (%)	STD (250µg/ ml)	Test sample (%)
100	82.60±0.20	40.90±0.16	77.18±0.27	42.08±0.21
200		48.23±0.51		47.35±0.94
300		60.12±0.46		53.32±0.46
400		58.27±0.33		57.28±0.80
500		56.14±0.38		55.04±0.08

Alpha-amylase inhibitory assay of *Aristolochia indica* methanolic extract were increased with the increasing concentration. It was concluded from the result that the methanolic extract exhibits higher alpha-amylase inhibitory activity at 60.12% at the concentration of 300µg/ml. The result was compared to the standard drug Acarbose which exhibits 82.60% inhibition. Alpha-glucosidase enzyme assay of *Aristolochia indica* methanolic extract were increased with the increasing concentration. It was concluded from the result that the methanolic extract exhibits higher alpha-glucosidase inhibitory activity at 57.28% at the concentration of 400µg/ml. The results were compared to the standard drug Acarbose which exhibits 77.18% inhibition

In the present study, the methanolic extract of whole plant of *Aristolochia indica* inhibited both the enzymes α-amylase and α-glucosidase. The result revealed that the methanolic extract of *Aristolochia indica* showed α-amylase and α-glucosidase inhibitory activity with increased concentration. Hence, themethanolic extract of *Aristolochia indica* can be used to reduce the risk of chronic disorders related with diabetes.

3.3 In vitro cytotoxicity (MTT)

Table 3. *In vitro* growth inhibitory activity (IC50 mg/ml) of HepG2 cell lines after treatment with concentrations of *A. indica* extract

S.No	Concentration mg/ml	Dilution	% cell viability	IC_{50}(mg/ml)
1	50	1:1	17.3	**57.4±1.41***
2	25	1:2	26.3	
3	12.5	1:4	49.8	
4	**6.25**	**1:8**	**57.1**	
5	3.12	1:16	62.4	
6	1.56	1:32	72.9	
7	Control	-	100	

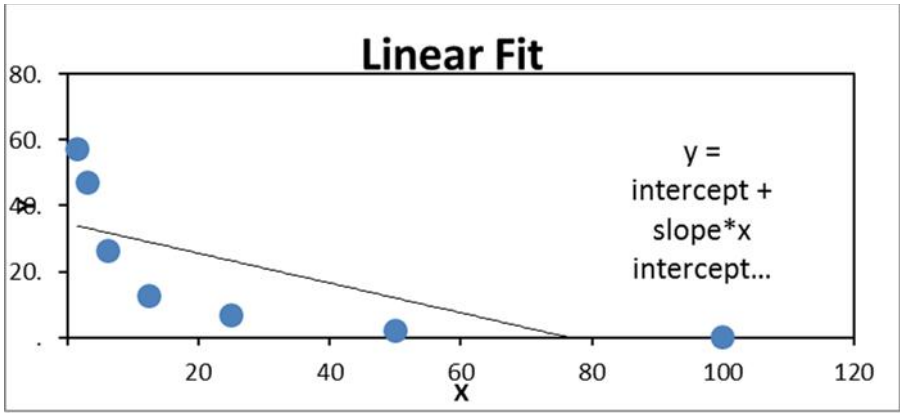


Figure 1 : IC_{50} Values of cell viability percentage on HepG2 cell line at various concentrations

The result showed that HepG2 cells proliferation was significantly inhibited by *A.indica* with an IC50 value of 6.25mg/ml of the concentration compare with normal cell inhibition.

Table 4: *In vitro* growth inhibitory activity (IC50 mg/ml) of VERO cell lines after treatment with concentrations of *A.indica* extract

S.No	Concentration mg/ml	Dilution	% cell viability	IC_{50}(mg/ml)
1	50	1:1	97	**49.5±2.61***
2	25	1:2	94	
3	12.5	1:4	92	
4	6.25	1:8	89	
5	3.12	1:16	86	
6	**1.56**	**1:32**	**84**	
7	Control	-	100	

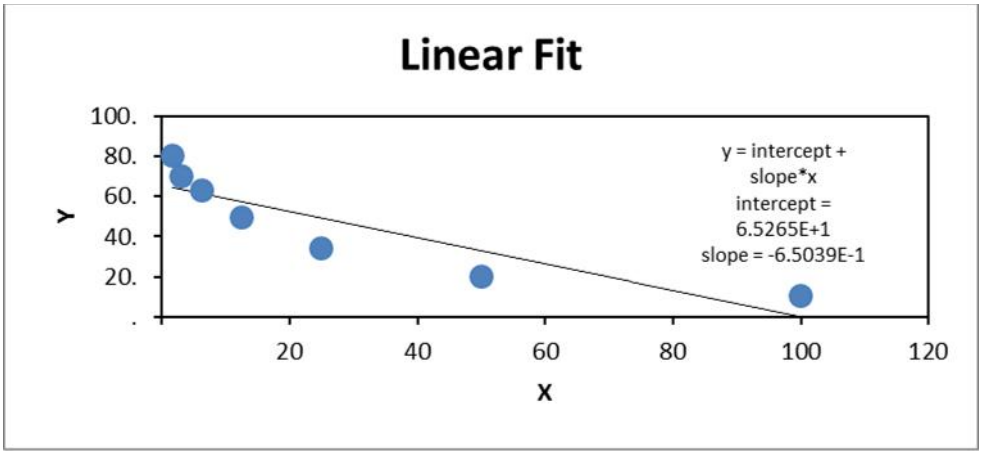
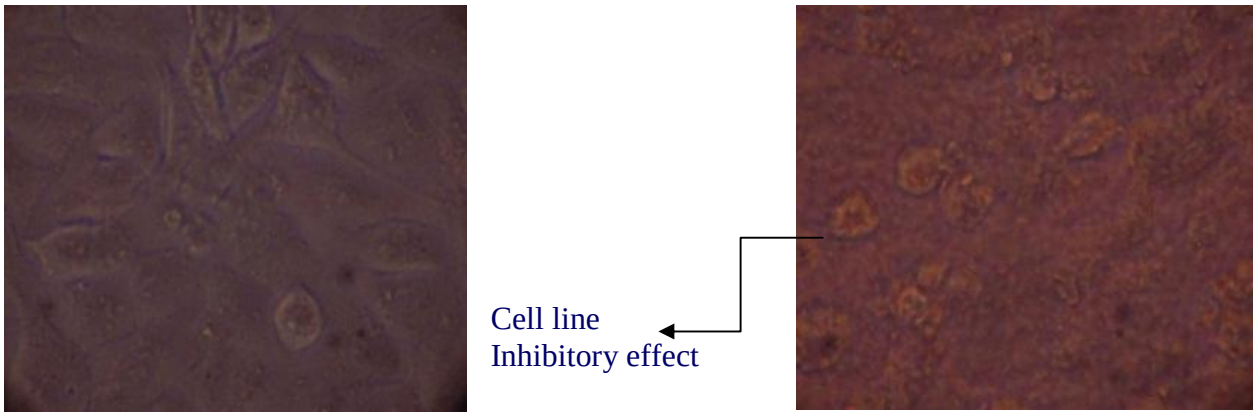


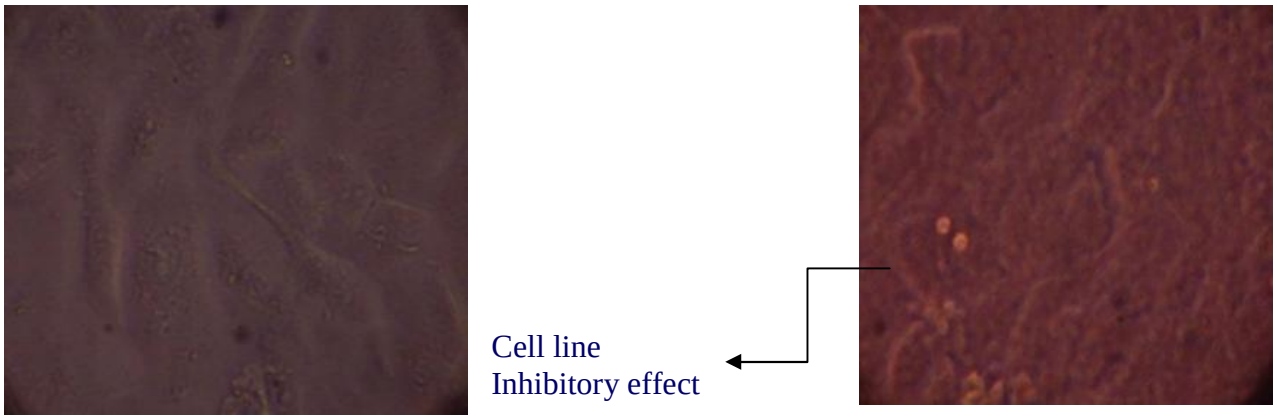
Figure 2 : IC_{50} Values of cell viability percentage on VERO Cell line at various concentrations

The non toxic dose of methanolic extract of A.indica on normal vero cell line showed that the percentage with regard to viability of cells was found to be 84% at a concentration of 50 mg/ml which decreased with increase in concentration



Control cell line 1.56mg/ml

Fig-3 Picture showed growth of cancer HepG2 cell lines (50-1.56mg/ml), and inhibition effect of cancer cell after 24 hrs at 6.25 mg/ml



Control cell line 1.56mg/ml

Fig 4. Picture showed growth of cancer HepG2 cell lines (50-1.56mg/ml), and inhibition effect of cancer cell after 24 hrs at 1.56mg/ml

4. Discussion

The *Aristolochia indica* L. is used to treat cholera, fever, bowel, troubles, ulcers, leprosy, poisonous bites (Achari et al, 1983), and also used as emmenagogue, abortificient, antineoplastic, antiseptic, anti-inflammatory, antibacterial, antioxidant and phospholipase A2 inhibitor (Chopra et al, 2006). The plant has been used in skin diseases. The fresh juices of the leaves are popular antidote to snake poison (Masud Rana et al, 2006). The leaves and bark are used in intermittent fever. It is used ethanomedically as an antitumor, anti-inflammatory, antibacterial and antioxidant and antimicrobial (Masud Rana et al 2006). The extracts are found have various medicinal activities like, immunomodulatory activity and antitumor activity (Mavundza et al, 2010).

In present study, *Aristolochia indica* in stabilization of the HRBC membrane and inhibiting albumin denaturation were determined. They showed the maximum membrane stabilization of 77.06% at the dose of 500µg/ml. Supporting the data, Seema Chaitanya Chippada *et al.,* (2011) reported the methanolic extract of *Centella asiatica*which showed the maximum membrane stabilization of 94.97% at the dose of 2000µg/ml. The result of the present study can be correlated with the previous report that the ethanolic extract of *Curcuma zedoaria* showed the maximum inhibition of protein denaturation of 77.15 at the dose of 500µg/ml which was reported by Arif Ullah, *et al.,* 2014. The maximum inhibition of protein denaturation was reported in the methanolic extract of *Piper betel* leaf (Muralinath, 2016). All the results were compared with the Diclofenac sodium standard which showed 89.74% protection.

The present study reveals tha, *Aristolochia indica* effectively inhibit both α-amylase and α-Glucosidase enzymes. the methanolic extract of dried whole plant powder *Aristolochia indica*inhibited both the enzymes alpha-amylase and alpha-glucosidase and the maximum inhibition was 60.12% at the concentration of 300µg/ml and 57.28% at the concentration of 400µg/ml respectively.

The major challenges associated with currently available anticancer agents include selectivity, toxicity, resistance, and development of a secondary malignancy. These drawbacks have motivated the search for newer, more efficacious, and better tolerated antitumor drugs, with natural products, especially plants, offering an inexhaustible reservoir for new drug discovery and development.

AnilKumar *et al.,* (2014) reported that the methanolic extracts of roots of *Aristolochia indica* was tested against cervical cancer cell lines (HeLa) and breast cancer cell lines (MDAM B 231) using MTT method. Vinodhini Subramaniyan *et al.,* (2015) also reported that chloroform and aqueous extract of leaves and of stem of *Aristolochia indica* was tested against human breast cancer cell line (MCF-7) by MTT assay.

5. Conclusion

To date, more and more metabolic diseases have influenced in human's health and quality of life. In the last few years, there has been a growing interest in the herbal medicine in care and management of diabetes both in developing and developed countries, due to their natural origin and less side effects. So, the of *A. indica* is the rich source of natural, which can be accounted for the traditional uses in prevention of diabetes and conservation of good health

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